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Improved Enumeration of Lactic Acid Bacteria in Mesophilic Dairy Starter Cultures by Using Multiplex Quantitative Real-Time PCR and Flow Cytometry-Fluorescence In Situ Hybridization

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Nucleic acid-based assays were developed to enumerate members of the three taxa *Lactococcus lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*, and *Leuconostoc* spp. in mesophilic starter cultures. To our knowledge the present is the first study to present a multiplex quantitative PCR (qPCR) strategy for the relative enumeration of bacteria. The multiplex qPCR strategy was designed to quantify the target DNA simultaneously relative to total bacterial DNA. The assay has a high discriminatory power and resolves concentration changes as low as 1.3-fold. The methodology was compared with flow cytometric fluorescence in situ hybridization (FLOW-FISH) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)–calcium citrate agar–based plate counting. For enumeration by FLOW-FISH, three new probes having the same specificity as the qPCR assay were designed and established. A combination with flow cytometry greatly reduced the time consumed compared to manual enumeration. Both qPCR and FLOW-FISH yielded similar community compositions for 10 complex starter cultures, with all detected subpopulations being highly significantly correlated ($P < 0.001$). Correlations between X-Gal–calcium citrate agar–based CFU and qPCR-derived counts were highly significant ($P < 0.01$ and $P < 0.001$, respectively) for the number of acidifiers versus *L. lactis* subsp. *cremoris* and for *Leuconostoc* spp. as quantified by the two techniques, respectively. This confirmed that most acidifiers in the studied PROBAT cultures are members of *L. lactis* subsp. *cremoris*. Quantitative real-time PCR and FLOW-FISH were found to be effective and accurate tools for the bacterial community analysis of complex starter cultures.

In many dairies, so-called complex or undefined multiple-strain cultures of lactic acid bacteria are extensively used as starter cultures. Compared to defined cultures, complex ones frequently offer advantages in terms of their technological properties, such as aroma formation and phage resistance. The balance between aroma- and acid-forming taxa is important for properties such as aroma production in butter and eye formation in cheese (30). Therefore, technologies are required to enumerate the abundance of key component bacteria. Conventional plate counting has been the chosen method in most microbiological laboratories. One of the major drawbacks of cultivation-based assays is their high time consumption of about 5 days. Several molecular biological techniques for community analysis have emerged over the past decade, and most take advantage of the molecular phylogeny derived from 16S rRNA comparative sequence analysis. For the detection and enumeration of probiotic strains, several previous studies have developed both real-time PCR assays and fluorescence in situ hybridization (FISH) probes (15, 16, 18, 23, 25, 29). However, only a few studies have developed quantitative real-time PCR assays for the enumeration of lactic acid bacteria used as starter cultures (12–14, 17).

Three taxa, *Leuconostoc* spp., *Lactococcus lactis* subsp. *cremoris*, and *L. lactis* subsp. *lactis*, are important components of many dairy cultures. Because of their different technological and enzymatic properties, the individual strains have different impacts on the properties of the final dairy product. Quantitative information about the composition of starter cultures is thus important to maintain or optimize the requested culture properties. In the present study, quantitative real-time PCR-based multiplex and flow cytometry-FISH (FLOW-FISH) assays were developed to allow a rapid, cost-effective, and quantitative determination of the three taxa described above.

MATERIALS AND METHODS

Blending of PROBAT-like cultures. Microbial strains were from the Danisco Global Culture Collection (DGCC) and Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ). Defined PROBAT-like cultures were created by mixing member strains of the three taxa *Leuconostoc mesenteroides* subsp. *cremoris* (DSMZ 20346^T and DGCC 8), *Lactococcus lactis* subsp. *lactis* (DGCC strains 113, 133, 456, 1212, and 1306), and *L. lactis* subsp. *cremoris* (DGCC strains 16, 111, 453, 563, and 1224). *Lactococcus* and *Leuconostoc* strains were grown overnight at 30°C in triple sugar tryptone broth [20 g/liter peptone from caseine, 5 g/liter yeast extract, 2.5 g/liter gelatin, 5 g/liter glucose, water free, 5 g/liter lactose EP, 5 g/liter sucrose, 4 g/liter NaCl, 1.5 g/liter sodium acetate · $3H_2O$, 0.5 g/liter L(+) ascorbic acid, 0.5 g/liter Tween 80] modified compared to the original reference (3) and MRS medium (9), respectively. Afterwards, 24 ml of each strain was pooled to obtain mixtures for each of the three taxa. These were then centrifuged at 4,500 × *g* for 5 min in a model 5804R centrifuge (Eppendorf, Hamburg, Germany) and resuspended in 1× phosphate-buffered saline (PBS) to achieve a twofold concentration. CFU were determined using modified triple sugar tryptone agar (modified triple sugar tryptone broth plus 15 g/liter agar, pH 7.0) and MRS agar. The preblends of the three taxa were mixed at different final concentrations to obtain different compositions.

Sample preparation and DNA isolation. Five-gram aliquots of direct-frozen PROBAT cultures were diluted in 40 ml of PBS and thawed at 4°C. The solutions were then adjusted to pH 7.0 using NaOH. Sodium citrate (solution of 40% [wt/vol]) was then added to a final concentration of 1%. The samples were mixed and incubated at 4°C for 30 min. Ten-milliliter aliquots of the solutions were centrifuged at 4,500 × *g* for 5 min. The cell pellets were washed three times with

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10 ml of 1× PBS and centrifuged as described before. Cell pellets of 0.5 to 1 g (fresh weight) were then subjected to DNA extraction as described below for the pure cultures, except that 5 ml of lysis buffer was used. All other volumes were adjusted accordingly.

Pure cultures were cultivated in liquid medium according to standard microbiological methods for lactic acid bacteria (triple sugar tryptone or MRS medium). Two to 5 milliliters of inoculated culture medium was incubated overnight at 30°C, and cells were concentrated by centrifugation at 4,500 × *g* for 5 min using a model 5804R centrifuge (Eppendorf, Hamburg, Germany). Cell pellets were processed immediately or stored at −20°C. For DNA extraction, they were resuspended in 180 μl lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme, 100 U/ml mutanolysin) and incubated for 30 min at 37°C. Afterwards, the DNeasy tissue kit (QIAGEN, Hilden, Germany) was applied according to the guidelines of the manufacturer to isolate DNA. In addition, the FastDNA SPIN kit for soil (Qbiogene, Heidelberg, Germany) and the Bilatest Bac kit (Bilatec, Viernheim, Germany) were used for some mixtures of strains. DNA concentrations were measured by absorbance using a BioPhotometer (Eppendorf, Hamburg, Germany) or applying the PicoGreen quantification assay (Molecular Probes, Eugene, OR).

X-Gal–calcium citrate agar for CFU determination. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)–calcium citrate agar (30) is used for the detection and differentiation of lactococci and leuconostoc species used in mesophilic cultures. The citrate-fermenting species of the genus *Leuconostoc* and the biovar *Lactococcus lactis* subsp. *lactis* bv. *diacetilactis* are defined as aroma formers in these mesophilic starter cultures. Because of their citrate fermentation, they form a clear zone on calcium citrate agar. For the discrimination of *Leuconostoc* spp. and *Lactococcus lactis* subsp. *lactis* bv. *diacetilactis*, the grown agar plates are covered with a layer of X-Gal (a lactose analogue). Several representative species of the genus *Leuconostoc* (i.e., *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*) hydrolyze X-Gal, releasing indigo blue. Hence, colonies appear greenish. Colonies of *Lactococcus lactis* subsp. *lactis* bv. *diacetilactis* remain white.

Colonies that do not form a clear zone do not ferment citrate and are interpreted as acidifiers in the complex mesophilic starter cultures. For PROBAT cultures, these are expected to comprise strains of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*.

Fluorescence in situ hybridization. Specific target sequences of the 16S rRNA of the taxa of interest were identified by use of the program package ARB (20). The specificities of the target sequences obtained were also checked using the PROBE_MATCH tool of the Ribosomal Database Study II (21). Probes CREM62 and LAC62 were deduced with the discriminating base being centrally located. Probe 68Rca (26) (equivalent to Llc of Beimfohr et al. [4]) has the discriminating base at the 3′ end, which is generally more difficult to discriminate by FISH. Previously developed probes targeting the 23S rRNA (4, 5) were not used because the scarcity of 23S rRNA sequences did not allow a reasonable validation of the target sequences. Pure cultures and cleared PROBAT cultures resuspended in PBS (see above) were fixed with 1 volume of ethanol (>96%) and stored at −18°C. For FISH, 3 μl from each fixed sample was spotted onto precleaned (washed in 1% HCl and 70% ethanol) and gelatin-coated [0.075% gelatin–0.01% $CrK(SO_4)_2$] slides. The slides were then dried at 46°C for 10 min. Following dehydration in 50, 80, and 96% ethanol for 3 min each, the samples were covered with 8 μl of hybridization buffer (22) and 1 μl of probe (50 ng/μl). Oligonucleotides were synthesized and fluorescently labeled with Cy3 or Oregon Green at the 5′ end by MWG Biotech (Ebersberg, Germany), Thermo Electron (Ulm, Germany), or Metabion (Planegg-Martinsried, Germany). Unlabeled helper probes (11) were designed when the fluorescence intensity conferred by rRNA-targeted probes was low. Probes and helper oligonucleotides were mixed at stock concentrations of 50 ng/μl for each oligonucleotide. A formamide gradient between 0 and 80% in the hybridization buffer was used to assess the optimal stringency for newly designed probes. Samples were hybridized at 46°C for 15 h in isotonically equilibrated humid chambers. Samples were subsequently treated with a posthybridization wash as described by Manz et al. (22) at 48°C for 15 min. Sodium chloride concentrations in the washing buffer were adjusted according to the formulas of Lathe (19). Slides were rinsed briefly with MilliQ water, air dried, and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA).

For optimization of the stringency for probes, fluorescence intensities of DGCC reference strains hybridized with EUB338 and newly designed probes were detected using an Olympus epifluorescence BX41 microscope equipped with a 100-W high-pressure mercury lamp, fluorescence filter sets (Olympus), and a 100× oil immersion lens. Hybridization stringencies were optimized based on visual inspection of hybridized DGCC reference strains. Microscopic images were acquired using a Color View digital camera (Soft Imaging System, Germany) and the analySIS software package (Soft Imaging System, Germany).

For FLOW-FISH analyses, Oregon Green-modified oligonucleotide probes were used. Hybridization conditions were identical to the ones applied for epifluorescence microscopy except that 20 to 50 μl of the ethanol-fixed cells was centrifuged at 4,500 × *g* for 5 min, washed once in 500 μl of 1× PBS, centrifuged, and resuspended in 100 μl of the appropriate hybridization buffer. A 12.5-μl aliquot of a solution containing probe and helper oligonucleotides (stock concentration, 50 ng/μl per oligonucleotide) was added and incubated (see above). Nonspecific staining was removed by incubating in 500 μl of washing buffer for 15 min (see above). Cells were finally resuspended in 100 μl of 1× PBS and stored on ice in the dark until flow cytometric measurements were performed.

Flow cytometry. Five to 20 μl of the sample was diluted in 500 μl FACS FLOW buffer (Becton Dickinson) to obtain a flow cytometric event rate of 500 to 1,000 s^{-1}. To differentiate between cells and debris, samples were stained with propidium iodide (PI; final concentration, 15 nM) for 5 min at room temperature. Samples were enumerated using a FACScan cytometer (Becton Dickinson) and the CellQuestPro software package (instrument settings: FSC, E01; SSC, 370; FL1, 710; FL3, 760; threshold SSC, 280). For each replicate, 30,000 events were enumerated. The following dot plots were recorded: FSC/SSC, FSC/FL1, and FSC/FL3. A region was defined around the PI-positive events within the FSC/FL3 plot. By gating the FSC/FL1 plot onto this region, only those cells conferring PI fluorescence were analyzed in terms of their probe-conferred Oregon Green fluorescence. A second region was then defined within the FSC/FL1 plot surrounding the Oregon Green-fluorescent, probe-labeled cells. The numbers of probe-labeled versus PI-positive events gave the relative proportion of each of the taxa. The percentages of the probe counts of LAC62, CREM62, and LEUC1026 were finally normalized relative to total bacterial percentages based on probe EUB338. For some strains and defined blends of strains, the proportions of living, injured, and dead cells were determined using the LIVE/DEAD *Bac*Light bacterial viability kit according to the guidelines of the manufacturer (Invitrogen, Carlsbad, CA).

Quantitative real-time PCR. Dual-labeled probes and unlabeled primers were synthesized by MWG Biotech (Ebersberg, Germany), Thermo Electron (Ulm, Germany), or Metabion (Planegg-Martinsried, Germany). All oligonucleotides used for quantitative PCR (qPCR) were diluted in ultraPure distilled water (Invitrogen) to stock concentrations of 100 μM. Multiplex PCRs contained 1× PCR buffer (QIAGEN, Hilden, Germany), 5 mM $MgCl_2$, 0.9 μM of each of the primers (Leuc986F, Bac1108R, LDH38F, and Lac159cR) (see Table 3, below), 0.3 μM of the primers Bac944F and Crem159R, 0.3 μM of the dual-labeled probe PLac72F, 0.2 μM of the dual-labeled probes PCrem72F, PBac1060R, and PLeuc1026F (see Table 3), 0.8 mM of each of the four deoxynucleoside triphosphates, and 2.5 U of HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany). PCR Mastermix aliquots of 16 μl per reaction mixture were dispensed into PCR tubes using a model CAS-1200 liquid handling system (Corbett Life Science, NSW, Sydney, Australia). Four microliters of template DNA of the approximate concentrations of 1 and 10 ng/μl was then added to the reaction tubes. The PCR was carried out on a Rotor-Gene model 3000 quantitative PCR cycler (Corbett Life Science), applying a first denaturation at 95°C for 15 min followed by 50 cycles at 94°C for 20 s and 58°C for 45 s. Fluorescence data were acquired at the end of each elongation step at 58°C in the four channels, FAM, JOE, ROX, and Cy5.

The data were analyzed using the Rotor-Gene software, version 6.0, and Microsoft Excel. C_T values (the threshold cycle) were determined by manually setting the threshold value at 0.03 in all fluorescence channels, applying dynamic tube normalization and slope correction. Efficiency values were determined using the comparative quantitation feature implemented in Rotor-Gene based on the second derivative of the quantification reactions. Reactions were performed at least in triplicate. For the calculation of relative proportions of each of the three taxa relative to DNA encoding bacterial 16S rRNA, the formula of Pfaffl (24) was applied:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T \text{ of target}}}{(E_{\text{ref}})^{\Delta C_T \text{ of ref}}} \quad (1)$$

where E is the efficiency, "target" represents the taxon of interest, "ref" is the reference gene total bacterial 16S rRNA, and ΔC_T is the difference in C_T for the "control" (the DNA of single strains of the target taxa of interest) minus the C_T for the "sample" (the DNA extracted from PROBAT or similar cultures). Ratios were expressed as percentages. Average efficiencies determined for all samples and controls in each of the channels were used for the calculations. This approach gave more consistent results than the calculation of ratios based on efficiency values for each single reaction because of the variability of efficiency

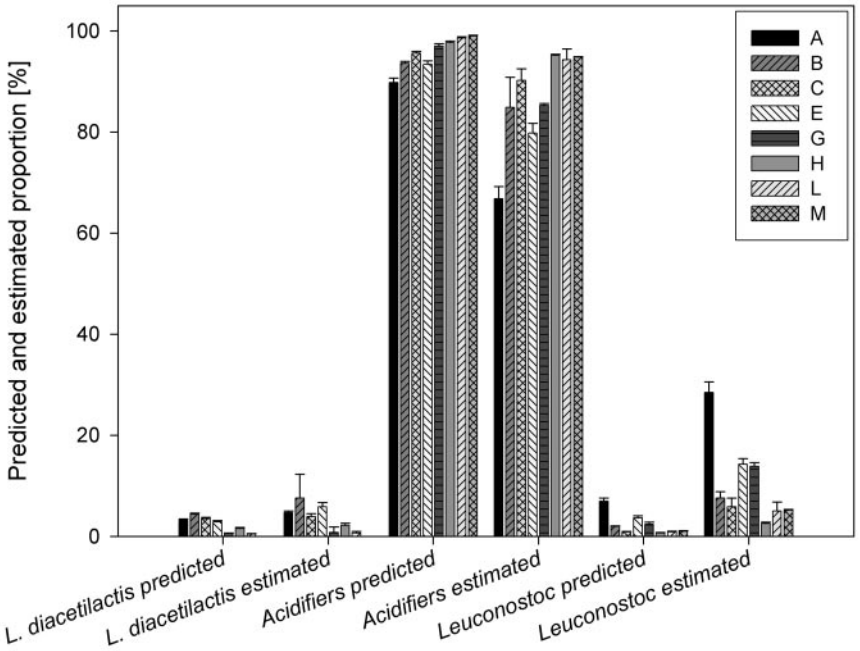

FIG. 1. Estimated and predicted relative proportions (percentages) of *L. lactis* subsp. *lactis* bv. *diacetilactis* (*L. diacetilactis*), acidifiers, and *Leuconostoc* spp. in eight defined PROBAT-like cultures (A through M) based on X-Gal–calcium citrate agar analysis versus predicted values based on CFU before blending of the individual taxa. Error bars indicate standard deviations of two replicate counts.

values determined by the second derivative model of the Rotor-Gene software (no data shown). For PROBAT samples, the sum of percentages for the three taxa, *Leuconostoc* spp., *Lactococcus lactis* subsp. *cremoris*, and *L. lactis* subsp. *lactis*, typically attained values between 100 and 125% relative to total bacterial DNA, since DNA of strains of the respective taxon was used as a control for the calculations of ratios (equation 1). Obviously, if all three taxa are present in one sample, as in PROBAT, competing PCRs will result in minor shifts of C_T values relative to the DNA of a single taxon. Therefore, all percentages were finally normalized to give 100%.

Statistical analysis. For the analysis of relationships between variables, Pearson product-moment correlation coefficients were calculated. Where the assumptions of normality and equal variance did not apply, the data were $\log_{10}$ transformed. Percentages were transformed by $\arcsin(x/100)^{1/2}$ before statistical analysis (27).

RESULTS

Application of X-Gal–calcium citrate agar plating to defined PROBAT-like cultures. The composition of the PROBAT-like cultures was predicted from CFU counts of the individual strains before blending and compared with the values estimated by X-Gal–calcium citrate agar enumeration after the blending (Fig. 1). A significant correlation between the estimated and predicted counts of acidifiers was found ($R^2 = 0.906$; $P < 0.01$). The slope of the linear regression being forced through the origin was merely 0.70, indicating that X-Gal–calcium citrate agar plating underestimated the actual CFU of acidifiers by about 30%. The estimated CFU of *Lactococcus lactis* subsp. *lactis* bv. *diacetilactis* also showed a significant correlation with the predicted values ($R^2 = 0.932$; $P < 0.01$) with the slope of the linear regression being 1.25, thus overestimating the actual CFU of this biovar by 25%. Moreover, there was a significant linear relationship between the estimated and predicted CFU ($R^2 = 0.953$; $P < 0.01$) for *Leuconostoc*; however, the X-Gal–calcium citrate agar method overestimated the CFU of this taxon about 3.5-fold (Fig. 1). Obviously, because of the significant overestimation of *Leuconostoc* by the X-Gal–calcium citrate agar method, skewed relative proportions of the three taxa compared to the predicted ones were obtained (Fig. 1). These PROBAT-like cultures were subsequently analyzed using the cultivation-independent techniques, and assays were developed as part of the present study.

Development of the multiplex qPCR assay. A multiplex qPCR assay was designed to quantify the DNA of the three taxa, *Leuconostoc* spp., *Lactococcus lactis* subsp. *lactis*, and *L. lactis* subsp. *cremoris*, relative to total bacterial DNA using four dual-labeled probes. After multiplexing and optimization took place, adjusting the concentrations of *Taq* DNA polymerase, deoxynucleoside triphosphates, and $MgCl_2$, C_T values showed virtually no shift in comparison to single and duplex reactions (data not shown). The data analysis resulted in percentages of the three taxa relative to total bacterial 16S rRNA. DNA of the individual taxa served as a control for which the C_T values were measured applying the same PCR conditions as for the analysis of defined or complex mixtures of the taxa. The calculated proportions of taxa were efficiency corrected using the efficiency values determined by the second derivative of the fluorescence increases (see Materials and Methods).

Resolution of the qPCR assay. A dilution series of DNA isolated from a PROBAT culture with increasingly narrower dilution steps (2-, 1.7-, 1.5-, 1.3-, and 1.1-fold corresponding to 50, 29.4, 19.6, 15.1, and 13.7% of the initial DNA concentration) was created to determine the maximum resolution of the multiplex qPCR assay (Fig. 2). It was apparent that even a 1.3-fold difference could be resolved in all four channels of the assay. This high resolution enabled the detection of small bac-

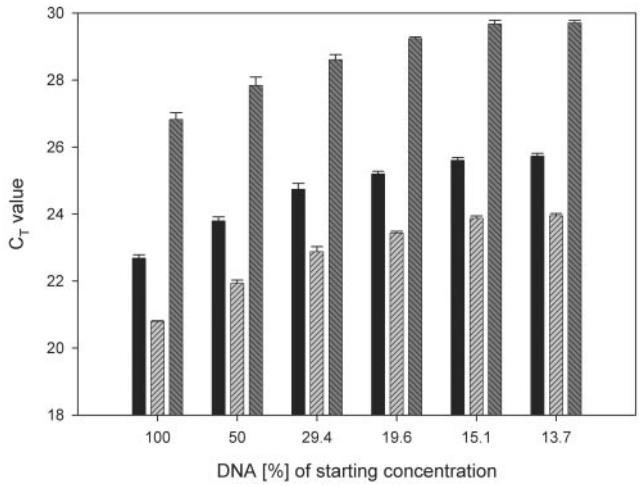

FIG. 2. Resolution of the multiplex qPCR assay, shown as C_T values versus DNA template concentrations in the *L. lactis* subsp. *lactis* (black bars), *L. lactis* subsp. *cremoris* (light gray bars), and *Leuconostoc* (dark gray bars) channels. DNA extracted from PROBAT with the DNeasy tissue kit was diluted 1:100 to obtain the starting concentration (100%). This was then further diluted at increasingly narrower steps, 2-, 1.7-, 1.5-, 1.3-, and 1.1-fold. Except for the pairwise comparison between the C_T values of the 1.3- versus the 1.1-fold dilution, values were significantly different (analysis of variance; $P < 0.05$). Error bars indicate standard deviations of C_T values of four replicates.

terial community changes well below a twofold concentration change in all four channels of the assay.

16S rRNA-targeting probes for FLOW-fluorescence in situ hybridization. Three new 16S rRNA-targeting probes were developed in the present study (Table 1). The specificities of the probes LAC62 and CREM62 were tested by hybridizing strains of both subspecies. At 10% formamide in the hybridization buffer, nonspecific fluorescence was in the background range. For LEUC1026, nontarget taxa such as *Listeria* spp. and *Enterococcus* spp. had at least two mismatches with the probe. These were thus not included for the tests. For two probes, helper oligonucleotides were developed to increase the otherwise low signal intensities (Table 1). For CREM62, helper oligonucleotides were necessary, although LAC62, targeting the same 16S rRNA region of the closely related subspecies, conferred relatively bright fluorescence without them.

All of the tested strains showed bright fluorescence after hybridization with probe EUB338, which is specific for *Bacteria* and was used as a positive control (Table 2). The specificities of the probes developed in the present study were as predicted in silico, although signal intensities were lower than that of EUB338 but within an acceptable range.

Application of the multiplex qPCR assay and FLOW-FISH to PROBAT-like cultures. The multiplex qPCR assay was applied to DNA extracts of defined PROBAT-like cultures that were also analyzed by X-Gal–calcium citrate agar (see above) and FISH analyses (see below). X-Gal–calcium citrate agar and the cultivation-independent assays differ in terms of their target taxa. Whereas X-Gal–calcium citrate agar cannot discriminate the two subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, the qPCR and FLOW-FISH assays developed in the present study discriminate them. Additionally, X-Gal–calcium citrate agar detects and differentiates the aroma-forming species of the genus *Leuconostoc* and *L. lactis* subsp. *lactis* bv. *diacetilactis* based on citrate fermentation and X-Gal cleavage. The molecular assays developed target the taxa *Leuconostoc* and *L. lactis* subsp. *lactis* as such (Tables 1 and 3). Hence, a comparison of X-Gal–calcium citrate agar and the cultivation-independent methods is possible if the component bacteria are known, as in defined cultures. Based on the CFU determined for the blends of the individual taxa, relative proportions were predicted. (Fig. 3).

Not only the qPCR assay but also the extraction efficiency of the applied DNA preparation protocol may have an impact on the estimated bacterial community composition. Therefore, three commercially available DNA extraction kits were compared in terms of the culture composition by qPCR (Fig. 4). The overall patterns of relative proportions across the eight different cultures were very similar to the ones predicted (cf. Fig. 3 and 4). Highly similar bacterial community patterns were observed for the different DNA extraction assays. However, there were some differences in terms of the comparison with the predicted values: in general the values for *L. lactis* subsp. *lactis* were lower than those predicted by CFU counts, with the lowest difference found for DNA extracted with the Bilatest Bac kit (Fig. 4C). The linear regression of the qPCR-based estimation for the latter kit versus the predicted values showed the greatest slope (0.774), thus underestimating the predicted relative proportion of *L. lactis* subsp. *lactis* by about 23%. The linear regressions of estimated versus predicted relative proportions of *L. lactis* subsp. *lactis* revealed high R^2 values, be-

TABLE 1. Details concerning the oligonucleotide probes used in the present study for FISH

Probe or oligonucleotide[a]	Target (position)[b]	Sequence (5′→3′)	Applied stringency (% formamide)	Reference
EUB338	Bacterial 16S rRNA (338–355)	GCTGCCTCCCGTAGGAGT	10	2
CREM62	*L. lactis* subsp. *cremoris* 16S rRNA (62–80)	CCAATCTTCATCGCTCAA	10	This study
H44	*L. lactis* subsp. *cremoris* 16S rRNA (44–61)	CTTGCATGTATTAGGCAC	10	This study
H81	*L. lactis* subsp. *cremoris* 16S rRNA (81–99)	TTCAAATYGGTGCAAGCA	10	This study
LAC62	*L. lactis* subsp. *lactis* 16S rRNA (62–80)	CCAACCTTCAGCGCTCAA	10	This study
LEUC1026	*Leuconostoc* spp. 16S rRNA (1026–1043)	CACTTTGTCTCCGAAGAG	10	This study
H1009	*Leuconostoc* spp. 16S rRNA (1009–1025)	AACACTTCTATCTCTAAA	10	This study
H1044	*Leuconostoc* spp. 16S rRNA (1044–1061)	CGACCATGCACCACCTGT	10	This study
NON338	Negative control	ACTCCTACGGGAGGCAGC	10	31

[a] Helper oligonucleotides are indicated by the prefix H.
[b] Base pair positions are based on *E. coli* numbering (7).

TABLE 2. Organisms used and results of FISH specificity tests

Taxon[a]	Origin and strain no.	FISH result with indicated probe[b]			
		EUB338	CREM62	LAC62	LEUC1026
L. lactis subsp. *cremoris*	DGCC 16	+	+	−	−
L. lactis subsp. *cremoris*	DGCC 36	+	+	−	−
L. lactis subsp. *cremoris*	DGCC 62	+	+	−	
L. lactis subsp. *cremoris*	DGCC 111	+	+	−	
L. lactis subsp. *cremoris*	DGCC 118	+	+	−	
L. lactis subsp. *cremoris*	DGCC 322	+	+	−	
L. lactis subsp. *cremoris*	DGCC 453	+	+	−	
L. lactis subsp. *cremoris*	DGCC 563	+	+	−	
L. lactis subsp. *cremoris*	DGCC 564	+	+	−	
L. lactis subsp. *cremoris*	DGCC 1137	+	+	−	−
L. lactis subsp. *cremoris*	DGCC 1138	+	+	−	
L. lactis subsp. *cremoris*	DGCC 1224	+	+	−	
L. lactis subsp. *lactis*	DGCC 113	+	−	+	
L. lactis subsp. *lactis*	DGCC 133	+	−	+	−
L. lactis subsp. *lactis* bv. *diacetilactis*	DGCC 136	+	−	+	−
L. lactis subsp. *lactis* bv. *diacetilactis*	DGCC 455	+	−	+	
L. lactis subsp. *lactis* bv. *diacetilactis*	DGCC 456	+	−	+	−
L. lactis subsp. *lactis*	DGCC 630	+	−	+	
L. lactis subsp. *lactis*	DGCC 648	+	−	+	
L. lactis subsp. *lactis* bv. *diacetilactis*	DGCC 740	+	−	+	−
L. lactis subsp. *lactis*	DGCC 773	+	−	+	
L. lactis subsp. *lactis*	DGCC 963	+	−	+	
L. lactis subsp. *lactis*	DGCC 964	+	−	+	
L. lactis subsp. *lactis*	DGCC 1212	+	−	+	
L. lactis subsp. *lactis*	DGCC 1306	+	−	+	
L. lactis subsp. *lactis*	DGCC 1449	+	−	+	
L. lactis subsp. *lactis*	DGCC 1547	+	−	+	
L. lactis subsp. *lactis*	DGCC 1609	+	−	+	
L. lactis subsp. *lactis* bv. *diacetilactis*	DGCC 1680	+	−	+	−
L. mesenteroides subsp. *cremoris*	DGCC 8	+	−	−	+
L. mesenteroides subsp. *cremoris*	DSMZ 20346^T	+	−	−	+
L. mesenteroides subsp. *mesenteroides*	DSMZ 20240	+	−	−	+

[a] Lactococci were identified based on their *ldh* and 16S rRNA gene sequences. *L. lactis* subsp. *lactis* bv. *diacetilactis* was identified based on its phenotype.
[b] The fluorescence signal was positive or negative; for LEUC1026, only selected nontarget strains of *L. lactis* were tested.

tween 0.964 and 0.994, for the three DNA extraction kits. Hence, bacterial community changes across the defined PROBAT-like cultures were well resolved by qPCR as predicted by CFU counts; however, there was a general tendency for the concentrations of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* to be lower and higher by qPCR, respectively. The qPCR assay is expected to detect DNA originating from both living and dead cells. However, only living cells would be expected to form colonies. Therefore, the blending of five PROBAT-like cultures was repeated and the CFU and the viability of the cells of the three taxa, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *Leuconostoc* spp., were determined. The CFU counts for the three taxa before blending were 1.1×10^8, 1.5×10^8, and 5.1×10^8 per ml, respectively. The numbers of viable cells determined with the Live/Dead kit were very similar, with 1.5×10^8, 1.8×10^8, and 5.9×10^8 per ml, respectively. The proportions of viable cells were 47, 70, and 24% for *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *Leuconostoc* spp., respectively. Hence, different proportions of the three taxa would be predicted if only living or also injured or dead cells were considered. The defined PROBAT-like cultures were then subjected to qPCR as described before. As expected, the derived percentages for the three taxa were highly correlated with the predicted proportions based on the number of total cells by flow cytometry ($r = 0.986$; $P < 0.001$)

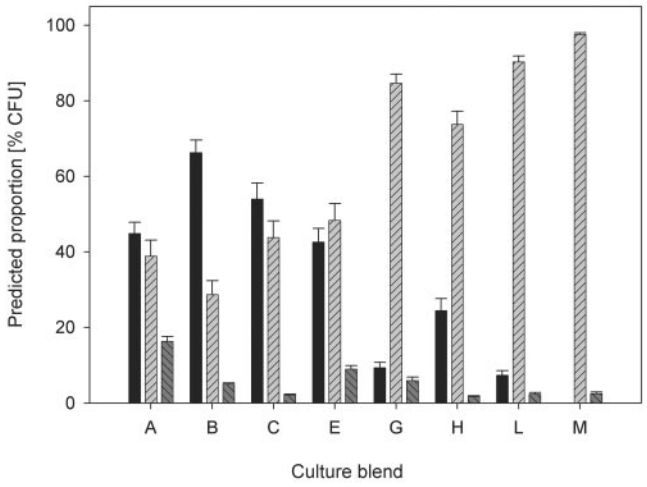

FIG. 3. Predicted compositions of eight defined PROBAT-like cultures (A through M) calculated from the percentages of CFU of blends of strains of the taxa. Black bars, *Lactococcus lactis* subsp. *lactis*; light gray bars, *L. lactis* subsp. *cremoris*; dark gray bars, *L. mesenteroides* subsp. *cremoris*. Data shown are mean percentages and standard deviations (error bars) of two replicate counts on triple sugar tryptone and MRS agar for lactococci and leuconostoc cells, respectively.

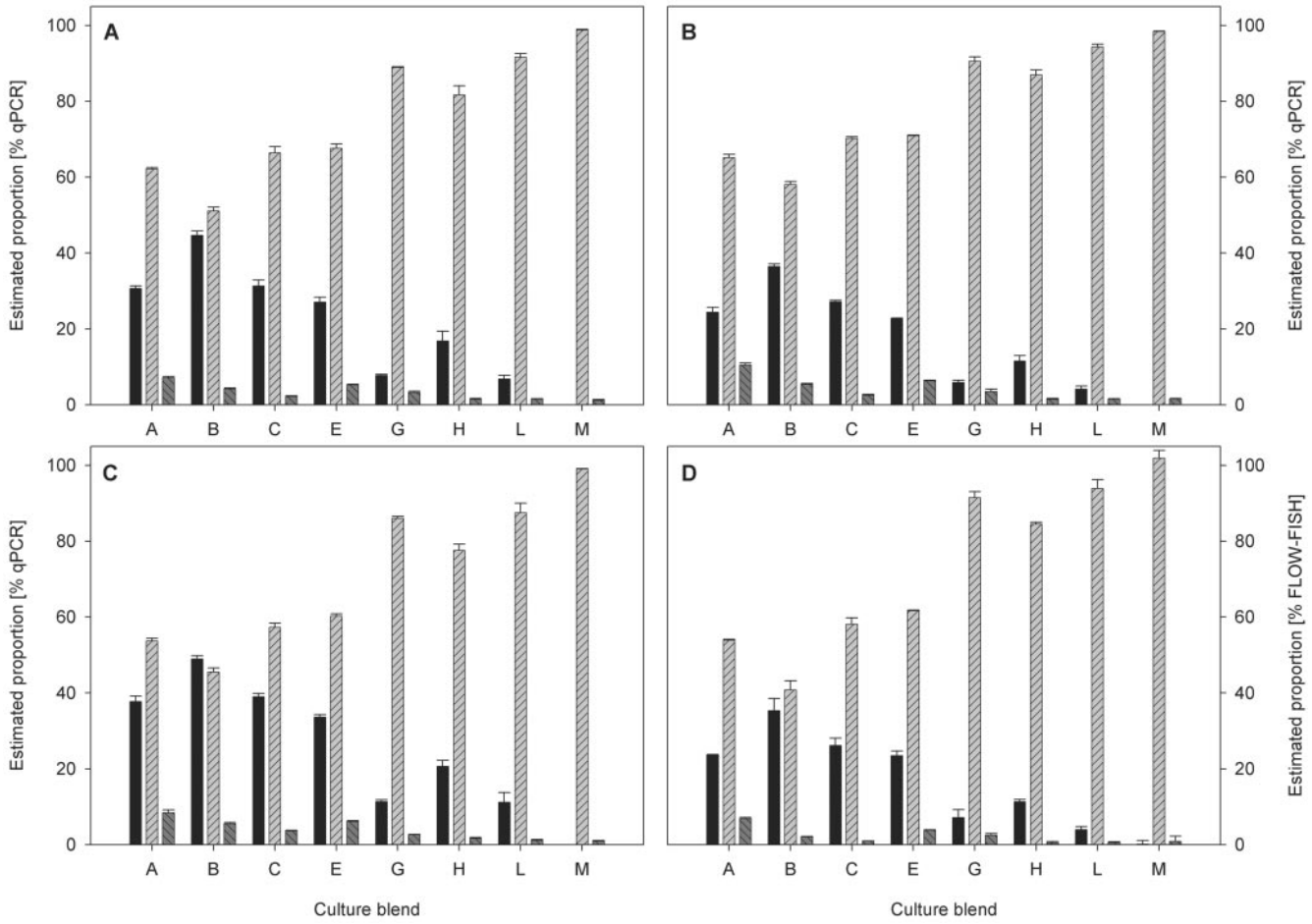

FIG. 4. Relative proportions (percentages) of the taxa *Lactococcus lactis* subsp. *lactis* (black bars), *L. lactis* subsp. *cremoris* (light gray bars), and *Leuconostoc mesenteroides* subsp. *cremoris* (dark gray bars) in eight defined PROBAT-like cultures. (A to C) Estimated relative proportions based on qPCR of DNA isolated with the DNeasy tissue (QIAGEN), FastDNA spin (Qbiogene), and Bilatest Bac (Bilatec) kits, respectively. (D) Values determined by FLOW-FISH. Error bars indicate standard deviations of three and two replicates for qPCR and FLOW-FISH, respectively.

and slightly weaker with the predictions based on the number of viable cells by flow cytometry ($r = 0.936$; $P < 0.001$). In addition to viability, also variable cell chain length would affect CFU counts and thus weaken relationships with nucleic-based quantification assays.

A similar composition of the defined PROBAT-like cultures as with qPCR was determined by FLOW-FISH enumeration (Fig. 4D). In fact, even minor changes of bacterial community composition across cultures were detectable by either of the two methods. In the present study the fluorescein analogue Oregon Green was used as the reporter for FISH oligonucleotide probes. However, although Oregon Green confers brighter fluorescence than fluorescein, it yields suboptimal fluorescence compared to the cyanine dyes Cy3 and Cy5, which could not be applied because of the available laser. Unfortunately, lower signal intensities of LAC62-conferred Oregon Green fluorescence did not allow a distinct separation of LAC62-positive populations from background signals in the defined cultures. This resulted in smaller detectable proportions for *L. lactis* subsp. *lactis* and explains why blends containing large portions of this subspecies (A through E) also showed the largest gaps between the detection rates of the bacterial probe EUB338 and the sum of the more specific ones, LAC62, CREM62, and LEUC1026, which were 15.4, 21.7, 14.7, and 11.0% for cultures A through E, respectively. Detection gaps were small or nonsignificant for cultures G through M. Overall, they correlated with the relative proportion of *L. lactis* subsp. *lactis* ($R^2 = 0.961$). This indicates that a portion of *L. lactis* subsp. *lactis* in these defined cultures conferred probe-bound Oregon Green fluorescence below the detection limit. Nevertheless, the proportions determined for *L. lactis* subsp. *lactis* by FLOW-FISH were similar to the ones determined by qPCR. The good agreement between predicted and estimated bacterial community composition formed a sound basis for the application of the qPCR multiplex and the FLOW-FISH assays to actual PROBAT cultures.

Bacterial community analysis of complex PROBAT cultures. Ten different PROBAT cultures were analyzed in terms of their bacterial community composition using X-Gal–calcium citrate agar–based plate counting and by the multiplex qPCR and FLOW-FISH assays developed in the present study. Pearson correlation coefficients were calculated to evaluate potential correlations between the counts obtained by the three enumeration techniques (Table 4). It was evident that qPCR-

TABLE 3. Primers and dual-labeled probes deduced in the present study[a]

Primer or probe[b]	Sequence (5′→3′)	5′ label[c]	3′ label[d]	Target
Bac944F	GGARCATGYGGHTTAATTCGA			Bacterial 16S rRNA gene
Bac1108R	CGCTCGTTGCGGGAC			Bacterial 16S rRNA gene
PBac1060R	CTCACGRCACGAGCTGACGACR	JOE	BHQ1	Bacterial 16S rRNA gene
Leuc986F	CAGGTCTTGACATCCTTTGAAG			*Leuconostoc* spp. 16S rRNA gene
PLeuc1026F	CTCTTCGGAGACAAAGTGACAGGT	Cy5	BHQ2	*Leuconostoc* spp. 16S rRNA gene
LDH38F	GTGACGGTGCTGTAGGTTC			*ldh* of *L. lactis* and other lactic acid bacteria
Crem159R	ATGAGAAAGGTCTTCTGCATCC			*L. lactis* subsp. *cremoris ldh*
Lac159cR	ATGAGAAAGGTCTTCTGCACCT			*L. lactis* subsp. *lactis ldh*
PCrem72F	CCTTGTTAACCAAGGAATTGCACAA	6-FAM	BHQ1	*L. lactis* subsp. *cremoris ldh*
PLac72F	TCTTGTAAACCAAGGGATTGCACAA	ROX	BHQ2	*L. lactis* subsp. *lactis ldh*

[a] Primers and probes developed for the multiplex quantitative real-time PCR assay for mesophilic dairy cultures in the present study targeted genes encoding 16S rRNA and lactate dehydrogenase.
[b] Dual-labeled probes carry the prefix P.
[c] Cy5 is a registered trademark of Amersham Biosciences Corp. Abbreviations: JOE, carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein; 6-FAM, 6-carboxyfluorescein; ROX, carboxy-X-rhodamine.
[d] BHQ1 and BHQ2 are registered trademarks of Biosearch Technologies, Inc.

based percentages of *L. lactis* subsp. *cremoris* were highly significantly correlated to the number of acidifiers as determined by X-Gal–calcium citrate agar analysis, and so was the correlation for *Leuconostoc* spp. X-Gal–calcium citrate counts of *L. lactis* subsp. *lactis* bv. *diacetilactis* were not significantly correlated to any of the other enumeration techniques. For qPCR versus FLOW-FISH, there were highly significant relationships between all of the identical subgroups, indicating that these methods yield equivalent results (Table 4).

DISCUSSION

X-Gal–calcium citrate agar plate counting of defined PROBAT-like cultures. Selective plating approaches are still the most widely used techniques for microbiological enumeration. For complex mesophilic cultures, X-Gal–calcium citrate agar can be regarded as a "gold standard" for the enumeration of the aroma- and acid-forming taxa in these cultures. In the present study the CFU determined for single strains that were then blended to obtain strain mixtures with compositions similar to complex mesophilic cultures were compared. We could show that X-Gal–calcium citrate agar underestimated the CFU of acidifiers relative to the predicted CFU by about 30%. For the aroma-forming taxa *L. lactis* subsp. *lactis* bv. *diacetilactis* and *Leuconostoc* spp., a significant overestimation was found (1.25- and 3.5-fold, respectively). Nevertheless, the CFU determined by X-Gal–calcium citrate were highly correlated to the predicted CFU. Hence, the results indicate that although the determination of the actual CFU may be inaccurate, X-Gal–calcium citrate agar can resolve changes of the composition of aroma- versus acid-forming taxa in these cultures with relatively good precision. It was also determined that a deviating response of the individual strains on X-Gal–calcium citrate agar could be an explanation for the observed over- or underestimation. The only deviation was found for *Leuconostoc mesenteroides* subsp. *cremoris* DSM 20346^T, which showed neither citrate fermentation nor X-Gal-hydrolyzing activity (three replicate analyses). This behavior of the strain was already shown previously (30). Therefore, this strain was not included for the comparison. In addition, for example, strains of the species *Leuconostoc pseudomesenteroides* also showed citrate fermentation and X-Gal-hydrolyzing activity (data not shown).

TABLE 4. Correlations between the three enumeration methods

Method and subpopulation	Correlation with qPCR results[a]		
	L. lactis subsp. *cremoris*	*L. lactis* subsp. *lactis*	*Leuconostoc* spp.
X-Gal–calcium citrate agar			
Acidifiers	**0.852****	−0.864**	−0.641*
L. lactis subsp. *lactis* bv. *diacetilactis*			
Leuconostoc spp.	−0.734*	0.682*	**0.896*****
FLOW-FISH			
L. lactis subsp. *cremoris*	**0.884*****	−0.884***	−0.766**
L. lactis subsp. *lactis*	−0.836**	**0.832****	0.766**
Leuconostoc spp.	−0.728*	0.687*	**0.870****

[a] Results (Pearson correlation coefficients of transformed percentages) for bacterial community analyses of 10 different PROBAT cultures based on the X-Gal–calcium citrate agar or FLOW-FISH method were compared to those based on qPCR. Correlations of equivalent subpopulations are shown in boldface. Only significant correlations are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Quantitative PCR-based quantification. As opposed to absolute quantification using standard curves of dilutions of target template DNA, a relative quantification strategy was followed in the present study. The reasons for doing so were that relative proportions rather than absolute numbers of the target taxa were of interest and that quantification relative to a reference gene is often advantageous in terms of the accuracy, as the creation of appropriate standards is laborious and critical (24). Thus, a multiplex qPCR assay was designed to quantify the DNA of the three taxa, *Leuconostoc* spp., *Lactococcus lactis* subsp. *lactis*, and *L. lactis* subsp. *cremoris*, relative to total bacterial DNA using four dual-labeled probes. For the discrimination of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, the lactate dehydrogenase-encoding genes were used instead of the 16S rRNA genes because of the higher sequence heterogeneity. The meaningfulness of the *ldh* gene to discriminate *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* has been shown previously (28).

Whereas the genome of *Lactococcus lactis* subsp. *lactis* IL-1403 contains six rRNA (*rrn*) operons, it contains only one *ldh*

operon (6, 10). The same number of *rrn* operons (six) has also been found for other lactococci and *Streptococcus thermophilus* (8). The different number of operons can be corrected for by applying isolated DNA of the different target taxa as a control (see equation 1, above). Since control DNA of different lactococci is used as a control, the difference in terms of copy numbers of rRNA and *ldh* genes does not have an impact on the results obtained. However, as total 16S rRNA serves as a reference gene for the calculation of percentages of lactococci and leuconostocs in PROBAT, different numbers of *rrn* operons between these two taxa would affect the results. In terms of representatives of the genus *Leuconostoc*, less information about the number of *rrn* operons is available. The draft genome of *Leuconostoc mesenteroides* ATCC 8293 contains three 16S rRNA-encoding genes (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi?page=taxonDetail&taxon_oid=400520000). *Lactobacillus plantarum* WCFS1 and *Lactobacillus johnsonii* NCC 533 have five and six *rrn* operons, respectively (10). Acinas et al. (1) showed for a number of representatives of the *Firmicutes* a range of four to seven *rrn* operons. Hence, a relative underestimation of leuconostocs cannot be ruled out if they possess fewer *rrn* operons than lactococci. That is, if there were a twofold difference in *rrn* operons, the theoretical underestimation would be close to a factor of 2 for leuconostocs if lactococci accounted for almost 100% in a culture, given the quantification regimen applied in the present study. However, FLOW-FISH- and qPCR-derived percentages for leuconostocs were similar for the defined culture blends (Fig. 4), thus not indicating an underestimation by qPCR in the present study.

Application of the multiplex qPCR and FLOW-FISH assays. The comparison of three commercial DNA extraction kits in terms of the culture composition by qPCR showed overall the same pattern across the eight different cultures. Thus, overall the commercial kits appeared to extract the DNA quantitatively from strains of the eight defined blends. In general, the values for *L. lactis* subsp. *lactis* were lower than the ones predicted by CFU counts, with the lowest difference found for DNA extracted with the Bilatest Bac kit. Hence, there was clearly an effect of the applied DNA extraction kit. These differences might be a consequence of the varied cell lysis efficiencies for different bacterial taxa.

Additionally, variable viabilities of cells would be expected to have an effect on CFU counts, since only living cells can form colonies on agar plates. It was shown that numbers of viable cells as determined with the Live/Dead kit and flow cytometry correlated well with CFU counts. For qPCR-derived percentages, a highly significant correlation with the percentages of total cells by flow cytometry ($r = 0.986$; $P < 0.001$), and a slightly weaker correlation with the predictions based on the number of viable cells ($r = 0.936$; $P < 0.001$) was found. Clearly, viability is a key criterion of starter cultures, since only viable cells are able to show the required metabolism. As DNA is known to be a relatively stable molecule, qPCR-derived data may not represent the viable fraction of microbial communities. Hence, if lactococci or leuconostocs show varied degrees of viability and cell lysis in PROBAT or similar cultures, qPCR-derived compositions may not be representative for the viable and active cells.

Highly significant correlations were found for the qPCR and FLOW-FISH results obtained for 10 different complex PROBAT cultures. Therefore, both nucleic acid-based detection and quantification techniques yielded virtually the same culture compositions ($r > 0.83$; $P < 0.001$). This confirmed the results obtained for the defined PROBAT-like cultures. Interestingly, qPCR and FLOW-FISH results were significantly correlated with the CFU counts obtained with X-Gal–calcium citrate agar in terms of the abundance of *L. lactis* subsp. *cremoris* and *Leuconostoc* spp., with the former being correlated to the CFU of acidifiers and the latter being correlated to the CFU of *Leuconostoc* spp. This former confirms the findings of previous isolation approaches (data not shown) that most acidifiers in the studied PROBAT cultures are members of *L. lactis* subsp. *cremoris*. The lack of a significant correlation between *L. lactis* subsp. *lactis* by qPCR and *L. lactis* subsp. *lactis* bv. *diacetilactis* by the X-Gal–calcium citrate agar method indicate an abundant presence of other nondiacetilactis strains of the subspecies.

In sum, the present study shows that both qPCR and FLOW-FISH are promising alternatives to conventional microbiological plate counting to enumerate mesophilic starter cultures. To our knowledge, this is the first study to present a multiplex qPCR strategy for the relative enumeration of bacteria. The applied relative quantification approach is an effective alternative to the laborious and often complicated use of absolute standard curves beyond the scope of expression studies in terms of qPCR. Compared to FLOW-FISH, qPCR has the advantage of being more flexible, since DNA and not rRNA serves as the target molecule. Thus, genetic differences found to discriminate groups of strains below the subspecies level can also be targeted by this technique. This avenue is currently being followed in our research labs.

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